Social isolation dysregulates endocrine and behavioral stress while increasing malignant burden of spontaneous mammary tumors

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In a life span study, we examined how the social environment regulates naturally occurring tumor development and malignancy in genetically prone Sprague–Dawley rats. We randomly assigned this gregarious species to live either alone or in groups of five female rats. Mammary tumor burden among social isolates increased to 84 times that of age-matched controls, as did malignancy, specifically a 3.3 relative risk for ductal carcinoma in situ and invasive ductal carcinoma, the most common early breast cancers in women. Importantly, isolation did not extend ovarian function in late middle age; in fact, isolated animals were exposed to lower levels of estrogen and progesterone in the middle-age period of mammary tumor growth, with unchanged tumor estrogen and progesterone receptor status. Isolates, however, did develop significant dysregulation of corticosterone responses to everyday stressors manifest in young adulthood, months before tumor development, and persisting into old age. Among isolates, corticosterone response to an acute stressor was enhanced and recovery was markedly delayed, each associated with increased mammary tumor progression. In addition to being stressed and tumor prone, an array of behavioral measures demonstrated that socially isolated females possessed an anxious, fearful, and vigilant phenotype. Our model provides a framework for studying the interaction of social neglect with genetic risk to identify mechanisms whereby psychosocial stressors increase growth and malignancy of breast cancer.

breast cancer | glucocorticoids | physiological stress | psychological stress | social behavior

A growing body of basic research and clinical studies suggest that stress and other psychosocial variables including low social support and chronic social isolation contribute to cancer progression (1–3). Specifically, dysregulation of the hypothalamic-pituitary-adrenal (HPA) axis is linked to breast cancer mortality, predicted by disrupted cortisol diurnal rhythms in women with metastatic disease (4).

Within a conceptualized framework of stress and disease, bio-behavioral factors are understood to influence multiple aspects of tumor growth including apoptosis, angiogenesis, invasion, and immunological escape to the metastatic cascade (5). As one example, transferring laboratory mice from group housing to social isolation accelerates growth of induced tumors and attenuates the effects of chemotherapy (6, 7). Prolonged exposure to the synthetic glucocorticoid dexamethasone has both mutagenic and clastogenic effects (8, 9). In the Sprague–Dawley rat model of naturally occurring breast cancer, the magnitude of the glucocorticoid stress response was associated with mammary tumor onset, whereas time to hormonal recovery from a stressor predicted growth rate of tumors (10).

Cellular mechanisms whereby the HPA axis could regulate cancer growth have been established in human breast cancer cell lines and in xenograft models. Glucocorticoid receptor (GR)-mediated survival mechanisms are induced by prolonged exposure to physiological concentrations of glucocorticoids and inhibited by GR specific antagonists (11). GR activation and resultant gene expression changes inhibit apoptosis in human breast cancer cell lines treated with clinically appropriate concentrations of a chemotherapeutic agent commonly used for treating human breast cancer—paclitaxel (12).

As in humans, animals living in the wild spontaneously develop benign and malignant tumors (13) and so may serve as a powerful model of the lifelong dynamic interplay between the psychosocial environment, physiology and genetic mechanisms that increase cancer risk. To date, most rodent models have been constrained by lack of facilities to adequately conduct life span studies of spontaneous tumors, and so are typically limited to the effects of acute or artificial stressors on carcinogen-induced tumors. During middle age, Norway rats spontaneously develop mammary tumors with a wide range of pathological diagnoses, ranging from benign fibroadenomas to invasive ductal carcinomas (14). Such rats provide an excellent model for a life span study of the effect of the social environment on stress vulnerability and spontaneous mammary tumor pathology (15).

Like humans, Norway rats are naturally gregarious, spend significant time in physical contact, form social relationships, and rear offspring cooperatively. In naturalistic settings, their burrow systems are a complex web of social interactions, including individuals that live apart from the group (16, 17). In laboratory settings, the costs of social isolation for female rats have proven to be high. Socially isolated female rats have a sustained and dysregulated glucocorticoid response to an acute stressor (18) and dysregulated cardiovascular responses to the everyday stressors of animal husbandry procedures (19). A life span study of sisters living in groups identified two independent and additive psychosocial risk factors associated with subsequent mammary tumor growth and mortality: an anxious temperament and failure to engage in reciprocal social contact during a stressor (20, 21).

The molecular genetics literature using transgenic, knockout and in vitro models points to a variety of gene candidates that could be affected by dysregulated stress responses to cause spontaneous tumors, for example, down-regulation of tumor suppressor genes such as *PTEN* or DNA repair genes such as *BRCA1*. Rapid growth could be mediated by up-regulation of genes involved in cell proliferation and/or cell survival, for example, *SGK1*, *MKP1*, *Myc*, and *AKT*. Some of these pathways are also regulated by ovarian steroids, although isolation accelerates reproductive senescence (22, 23), suggesting that estrogen and progesterone receptor status

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might not be associated with tumor burden in middle-aged and socially isolated female rats.

Keeping potential genetic targets in mind, our goal is to determine whether social isolation dysregulates glucocorticoid stress responses across the life span and increases glucocorticoid receptor activity in nuclei of mammary tumor cells. This would be a potential downward causal pathway along which chronic social stressors increase risk for mammary cancer. Indeed, glucocorticoids have been shown in cell lines to down-regulate expression of the important human tumor suppressor gene, *BRCA1* in breast cells, potentially leading to increased malignant transformation (24).

Here, we randomly assigned genetically comparable female rats (99% inbred strain) to two social conditions: living in a social group or living alone. We test the hypothesis that social isolation is associated with dysregulation of endocrine and behavioral responses to stress detectable early in adulthood, months before tumorigenesis. We further hypothesize that the accumulated effects of dysregulated stress responses typical of social isolates would affect multiple basic tumor characteristics, namely onset, mass, multiplicity, location, and malignancy.

To verify that lifelong isolation was associated with dysregulated hormonal and behavioral stress, we examined the basal and reactive functions of the adrenal axis at puberty and middle age to determine whether or not rats maintain characteristic differences in the magnitude of stress reactivity throughout adulthood and whether adrenal dysfunction is subsequently associated with development of mammary tumors. We had already established a correlation between animals with fearful temperament and mammary tumor burden and death (20). Here, we establish a causal relationship between affect and disease by manipulating the social context and creating the fearful, anxious, and tumor-prone phenotype through random assignment to isolate housing.

Results

Mammary Tumors. ***Tumor burden.*** By middle age (15.1 ± 0.1 months), 74% of female rats, whether group housed or isolated, had developed spontaneous mammary tumors detectable by palpation. Socially isolated females, however, had a tumor burden 84 times that of age matched controls living in groups (isolated = 27.17 ± 14.99 gm vs. grouped = 0.32 ± 0.12 gm; log transformed weights, $P \leq 0.04$; Fig. 1*A*). Although incidence of developing at least one palpable mass was similar in the two social conditions (relative risk 1.11; 78% isolated rats vs. 70% group housed rats, Fisher Exact, $P = 0.72$), isolation increased the number of discrete tumor masses by 135% (isolated = 4.7 ± 1.4 tumors, grouped = 2.0 ± 0.5 tumors, $t = 2.2$, $P \leq 0.05$). Among isolates, tumors were more widespread, developing in three if not all four mammary quadrants (left and right, thoracic and inguinal; each quadrant contains three glands); the tumors of all group-housed rats were confined to one quadrant (Fisher's Exact, $P = 0.03$). Regardless of tumor burden, imposed social isolation did not affect body weight [social condition F (1, 32) = 1.34, NS; tumor burden F (1, 32) = 0.32, NS; interaction F (1, 32) = 1.3, NS].

Tumor diagnosis. The naturally occurring tumors were diverse histological types (Fig. 1 *B–H*), including malignant tumors (36%: invasive ductal carcinoma, ductal carcinoma in situ, premalignant intraductal hyperplasia, and fibrosarcoma) and benign tumors (64%: fibroadenoma, intraductal papilloma, and lactacting hyperplasia and adenoma). The majority (63%) were epithelial in origin, 26% were stromal and 11% a mixture of hyperplastic epithelial and stromal cells. Ductal carcinoma in situ (DCIS) and invasive ductal carcinoma (IDC) were the most prevalent malignant tumors (90%). IDCs were the largest (33.6 ± 16.7 g; all post-hoc P values ≤ 0.01), micromasses the smallest (0.11 ± 0.02 g; all post-hoc P values ≤ 0.01); DCIS and benign tumors were of similar intermediate weight [17.0 ± 11.8 g, 15.7 ± 15.3 g; post-hoc NS; one-way analysis of variance F (3, 80) = 10.7, $P \leq 0.0001$].

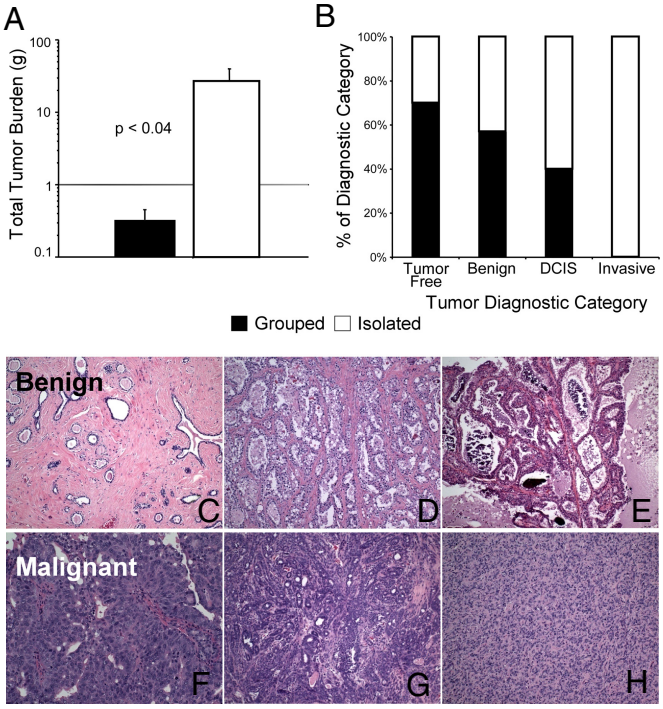

Fig. 1. Effect of long-term social isolation on mammary tumor growth and diagnosis at 15 months. (*A*) Tumor burden (mean ± SEM) after living alone or in noncrowded social groups. (*B*) Rats with ductal carcinoma in situ (DCIS) or malignant tumors were primarily socially isolated; those with benign or no tumors lived in groups. (*C–H*) Wide range of naturally developing mammary tumors: (*C*) fibroadenoma; (*D*) lactating adenoma; (*E*) intraductal papilloma; (*F*) DCIS; (*G*) invasive ductal carcinoma; (*H*) fibrosarcoma.

Isolation and malignancy. Isolation increased the development of malignant tumors (Fig. 1*B*), which naturally developed in all four mammary gland quadrants [social condition F (1, 51) = 8.96, $P \leq 0.01$, quadrant F (3, 51) = 8.80, $P \leq 0.0001$; interaction F (3, 51) = 3.37, ≤ 0.03, repeated measures (mammary gland quadrant) ANOVA]. Isolated animals had a 3.3-fold relative risk of developing at least one mammary carcinoma; fully 50.0% had IDC, DCIS, or a pre-DCIS tumor in sharp contrast to group housed females, where their incidence was only 15.4%.

Lifelong Housing Effects on Stress Responses. ***Response to predator odor at 3 months of age.*** Compared to those in stable social groups, female rats that were socially isolated from 1 to 3 months of age had a larger corticosterone response, measured 30 min after exposure to a novel cage scented with fox urine (isolated increased 9.2 ± 2.3 μg/dL, grouped increased 0.9 ± 2.4 μg/dL; $P \leq 0.02$; see Fig. 2*A*). Thus, random assignment to social isolation, rather than group living, increased the corticosterone responses to a natural psychological stressor (predator odor) by 10-fold.

Response to physical stressor at 13 months of age. After 12 months of isolation and before mammary tumors were palpable, rats developed basal hypocortisolemia, with low baseline levels of corticosterone, in comparison with group-housed rats (see Fig. 2*B*; $t = 3.38$, $P \leq 0.002$). They also had larger corticosterone response to a 0.5-h of physical restraint, a stressor that simulates a burrow collapsing (see Fig. 2*C*, reactive corticosterone at 30 min adjusted for baseline levels: isolated = 68.5 ± 5.4 μg/dL change from baseline, grouped = 50.4 ± 6.8 μg/dL; $t = 2.0$, $P \leq 0.05$). From baseline, their corticosterone rose 10.2 ± 2.7-fold within 30 min and continued to rise after the stressor ended, whereas corticosterone in group housed animals rose only 2.6 ± 0.9-fold ($t = 2.8$, $P \leq 0.01$). In addition to differences in basal and reactive corticosterone levels,

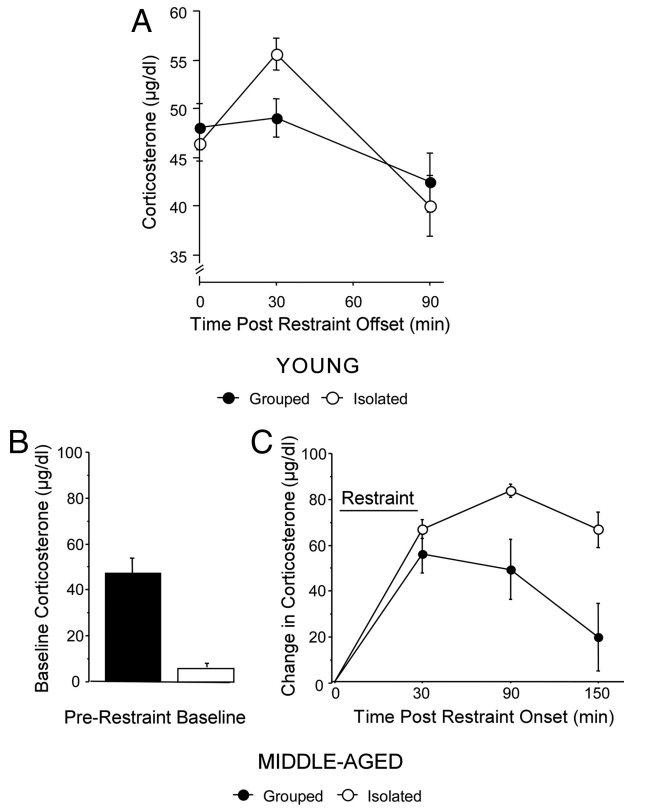

Fig. 2. Corticosterone dysregulation evident in young socially isolated females (3 months old) and severe by middle age (13 months old). (*A*) High corticosterone response and normal recovery (mean ± SEM) after an acute stressor, predator odor. (*B*) Low baseline corticosterone at the diurnal rhythm nadir. (*C*) A high prolonged corticosterone response, still rising from baseline 1 h poststressor, and delayed recovery 2 h post-stressor.

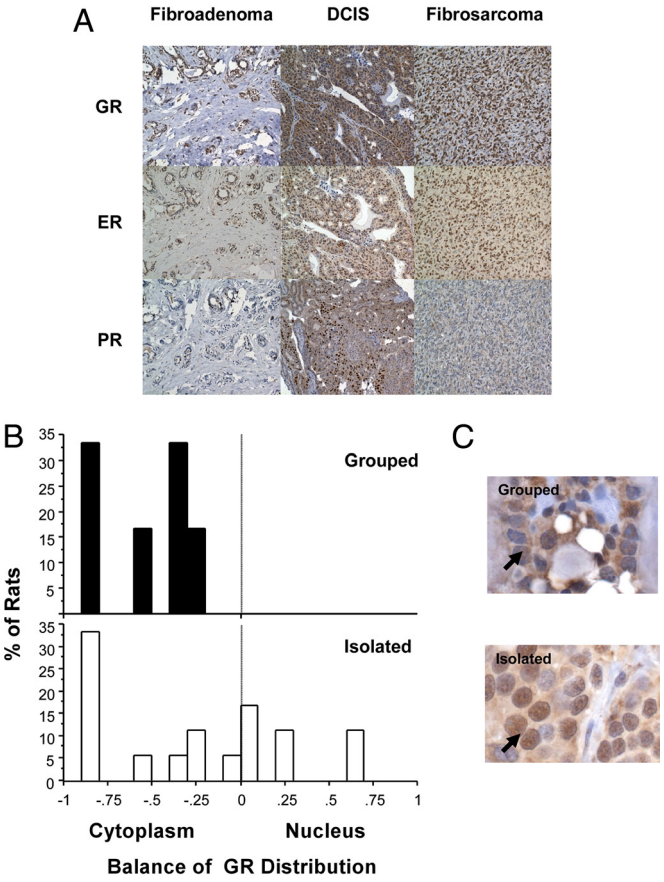

Fig. 3. Glucocorticoid (GR), estrogen (ER), and progesterone (PR) receptor status (brown stain) in different mammary tumor types. (*A*) Fibroadenoma: GR+ in epithelial and stromal cells, ER+ and PR+ only in epithelial cells. DCIS: GR+, ER+ and PR+. Fibrosarcoma, GR+ and ER+, but PR− . (*B*) Isolation and the relative distribution of GR (+ = nucleus vs. − = cytoplasm). (*C*) Exemplars: grouped: more cytoplasmic GR (brown surround-blue center); isolates: more nuclear GR (brown center-blue surround).

isolates recovered hormonally from this stressor more slowly (Recovery z Score: isolated = −0.36 ± 0.23, grouped = +0.25 ± .17, $t = 2.1$, $P \leq 0.04$), demonstrating that isolated animals sustained higher circulating levels of glucocorticoid 2 h after the stressor had ended. Taken together, these data indicate that before tumor development, socially isolated rats were exposed throughout adulthood to higher and more prolonged corticosterone in response to experimental stressors.

The dynamics of the corticosterone response to an acute stressor predicted the mammary tumor burden measured 2 months later [multiple regression F (4, 22) = 5.89, $r = 0.75$; $P \leq 0.003$; social condition ($\beta = -2.4$, $t = 2.9$, $P \leq 0.01$)]. Both high corticosterone at the end of the stressor and slow recovery to baseline predicted a larger tumor burden [corticosterone reactivity (log rise as proportion of baseline) $\beta = -2.8$, $t = 2.4$, $P \leq 0.03$; corticosterone recovery (z score) $\beta = -2.0$, $t = 4.0$, $P \leq 0.001$]. Baseline values of stress hormone, in this model, had no significant effect (baseline, $\beta = 0.0$, $t = 0.5$, NS).

Glucocorticoid Receptor Status of Mammary Tumors and Corticosterone Dynamics. To determine whether glucocorticoid receptors (GR) were expressed in mammary gland tumor tissue of rats, we performed immunohistochemistry and found that indeed, rat mammary tumors, including benign fibroadenomas, DCIS, and IDC all expressed the GR (Fig. 3 *A–C*), demonstrating the capacity of mammary tumor cells to respond to corticosterone.

Mammary gland tumors can arise from ductal epithelial cells, both basal and luminal, as well as from stromal cells, which are found in the mammary connective tissue. First, we assessed distribution of GR within the cells of each type of tissue (i.e., cytoplasmic and nuclear). In the epithelial tumor tissue, GR was found in the nucleus and the cytoplasm, although more cells had GR in the cytoplasm (75.0 ± 0.1% of cells) than in the nuclei (29.2 ± 0.1% of cells). Mammary stromal cells had much lower levels of GR in both intracellular locations (cytoplasm, 20% of cells; nuclei, 16% of cells).

Among socially isolated animals, the GR was more likely to be found in the nucleus compared to the cytoplasm in the tumor sample (Fig. 3 *B* and *C*; 44% of isolated females vs. 0% of grouped females, $X^2 = 4.0$, $P \leq 0.05$), indicative of dynamic translocation rather than steady receptor state. Nuclear translocation is typical of ligand-bound GR and demonstrates the potential for regulation of gene expression.

Lifelong Housing Effects on Ovarian Function and Tumor Status. If the middle-aged ovary mediates the effects of isolation on mammary tumor growth and malignancy via estrogen or progesterone receptor activation (25), then we would expect isolation to be associated with more hormonally active ovaries and/or perhaps, increased ER and PR expression in tumor epithelial cells. In fact, mammary tumors from isolated female rats grew in the milieu of early senescent ovaries, with only secondary and atretic follicles at necropsy, while mammary tissue from group-housed rats continued to be exposed to hormonally active ovaries, including ovulatory follicles (estrogen) and corpora lutea (progesterone) (23). To

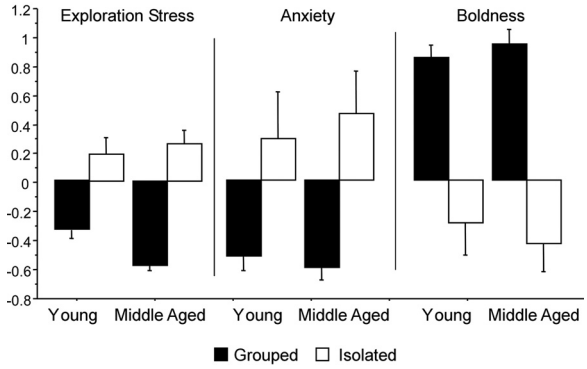

Fig. 4. Social isolation exacerbated three types of behavioral stress responses (age specific z-score mean ± SEM.) assessed in young adulthood (5 months) and repeated in middle age (15 months). Exploration Stress: Social Condition $F(1,36) = 52.1$, $P \leq 0.0001$; Age $F(1,36) = 1.8$, NS; Interaction $F(1,36) = 4.8$, $P \leq 0.03$ (Isolated animals did not improve with age as did those in groups.) Anxiety: Social Condition $F(1,34) = 13.80$, $P \leq 0.001$; Age $F(1,34) = 0.1$, NS; Interaction $F(1,34) = 0.0$, NS. Boldness: Social Condition $F(1,34) = 57.5$, $P \leq 0.0001$; Age $F(1,34) = 0.0$, NS; Interaction $F(1,34) = 0.2$, NS.

confirm inferences from these anatomical cross-sectional data, we determined that estrogen exposure throughout the four months before tumor diagnosis did not predict tumor burden ($r = 0.02, P \leq 0.95$; bioassay, estrogenization of vaginal epithelium).

The expression of nuclear ER and PR expression by immunohistochemistry in mammary tumor cells were highly correlated ($r = 0.79, P \leq 0.0001$). Most tumors were ER^+PR^+ (60% of cells with nuclear staining (Fig. 3*A*). The remainder was ER^-PR^- (30%) or positive for only ER or PR [ER^+PR^- (5%); ER^-PR^+ (5%)]. Malignant tumors had more ER-positive cells than did benign tumors (48.5 ± 9.5% vs. 11.0 ± 6.9%), and tended to have more PR-positive cells [27.5% ± 8.0% vs. 15.0 ± 5.5%; Diagnosis F (1, 18) = 5.8, $P \leq 0.03$; Receptor type F (1, 18) = 1.2, $P \leq 0.30$, interaction F (1, 18) = 5.7, $P \leq 0.03$, repeated measures (ER and PR in each tumor) ANOVA].

Nonetheless, social condition was not associated with nuclear staining for ER or PR [social condition F (1, 18) = 0.31, NS; receptor type F (1, 18) = 0.26, NS; interaction of social condition and receptor type F (1, 18) = 0.93, NS]. Finally, none of these indicators of ovarian function and ER/PR status were associated with stress reactivity or recovery, measured hormonally or behaviorally, or with GR status (NS, $0.28 \leq$ all P values ≤ 0.88).

Lifelong Housing Effects on Behavior: Routine Stressors Assessed at 5, 10, and 15 Months of Age. Social isolation also increased anxiety and reduced boldness, measured during an exploration stressor at both 5 and 15 months of age. When placed in the home corner of this novel exploration arena, isolated females did not move for prolonged times, and then proceeded slowly only in tight contact with the walls, exploring few new areas (Fig. 4). At both ages, isolated female displayed more species-typic anxiety behaviors during exploration: freezing, piloerection, urination, or defecation, which correlated with their level of Exploration Stress (Fig. 4; Anxiety z Score and Exploration Stress z Score: 5 month $r = +0.51$, $P \leq 0.0001$; 15 month $r = +0.60, P \leq 0.0001$). In contrast, group housed animals, explored more at both 5 and 15 months of age, crossing the open field, the most threatening part of the environment and displaying more boldness [e.g., rearing on their hind legs, standing, a steady, constant gait (Fig. 4); Boldness z and Exploration Stress z: 5 months $r = -0.49, P \leq 0.002$; 15 months $r = -0.78, P \leq 0.0001$].

The emotional effects of social isolation persisted within individual rats between 5 and 15 months of age (Exploration Stress z scores $r = +0.51$, $P \leq 0.0001$; Anxiety z Scores $r = +0.35$, $P \leq 0.04$; Boldness z Scores $r = +0.46$, $P \leq 0.004$). The level of Exploration Stress (ES) also predicted level of vigilance and anxiety after opening the home cage, a routine daily husbandry practice [latency of high ES rats to emerge = 54.2 ± 17.2 s., average to low ES rats = 27.4 ± 14.1 s, Logrank (Mantel-Cox) $X^2 = 4.77, P \leq 0.03$]. Responses to this everyday practice also persisted throughout middle age, even after 300 and 450 repetitions (10 and 16 months of age, $r = +0.51$, $P \leq 0.0001$; $r = +0.88$, $P \leq 0.0001$).

Association Among Manipulated Social Environment, Subsequent Psychoendocrine Stress, and Tumor Growth. To assess the coherence of psychoendocrine variables and disease outcomes within individual animals, we conducted a confirmatory factor analysis. As expected, key variables were significantly associated with each other, and contributed to a single factor measured at multiple levels of organization (orthogonal or varimax rotation, Eigen Value = 2.3, 55% of variance, $P < 0.01$; coefficients of shared variance with a single factor: social isolation 0.92, anxiety, fearfulness and vigilance 0.82, prolonged glucocorticoid stress recovery 0.55, and tumor burden 0.63). These associated variables may be candidates for a causal cascade, given their sequential development during a longitudinal experiment: social isolation, dysregulated hormonal and behavioral stress responses, and mammary tumor progression.

Discussion

In these studies, we show that female rats living in social isolation from puberty through late middle age became progressively more reactive to superimposed acute stress, first developing a heightened, and ultimately a prolonged, corticosterone stress response to either brief predatory odor or restraint stress. By randomly assigning female Sprague–Dawley rats to social isolation, we also reveal the importance of psychosocial modulation of a heritable risk for tumor development, because social isolation increased the size, number, distribution, and malignancy of spontaneous mammary tumors.

Interestingly, the magnitude of social isolation's effect on several characteristics of mammary neoplasia—135% increase in number, 8,391% increase in size and a 3.3-fold increase in relative risk of malignancy—is significantly greater than that of unlimited-access to food versus an energy-restricted diet. Prior to the current study, widely documented as the greatest environmental modulator of mammary tumor development in rodents (26). By comparison, unlimited access to high metabolizable energy food increased tumor incidence by only 90%, produced a modest 1.33 relative risk for malignancy, and had no effect on tumor growth (27).

Beginning in early adulthood and continuing throughout midlife of the female rat, we found that the adrenal axis of socially isolated animal was dysregulated, first manifesting as a higher corticosterone response and ultimately as markedly low baseline corticosterone levels indicative of hypocortisolemia (28). This was followed by high and sustained levels of corticosterone in response to a moderate stressor. This last pattern typically reflects damage and aging of the hippocampal system, as well as effects of vasopressin, corticotropin-releasing hormone and pro-opiomelanocortin-derived peptides regulating the adrenal axis (29). Isolation also induced glucocorticoid hyperresponsiveness in young adulthood months before mammary tumors developed in late middle age. Finally, mammary tumors reduce, rather than augment, glucocorticoid reactivity in rats (30), contravening the converse hypothesis that tumor development was itself stressful and caused the observed hyperreactivity.

Given the mild, yet repetitious, everyday stressors of laboratory life (19), the isolated rats and their mammary tissues were likely exposed throughout adulthood to prolonged pulses of higher levels of corticosterone with relatively low corticosterone levels between stressful events; the low basal levels could also feedback to increase steady-state glucocorticoid receptor (GR) expression allowing a highly robust intermittent response to acute stressor-induced glucocorticoids (31). This pattern of hypocortisolemia and prolonged elevated corticosterone in response to an intermittent acute stressor

may contribute to tumor initiation and growth, since GR activation has been associated with anti-apoptotic signaling in epithelial cells, including malignant human breast epithelial cells and premalignant mammary epithelial cells such as MCF10A-Myc cells (11).

Here, we also demonstrate that in social isolation, mammary gland GR was more commonly found in the nucleus. While in lymphocytes, activation and translocation of the ligand-bound receptor to the nucleus is associated with cell death (32), in cultured malignant human breast epithelial cells, glucocorticoid-mediated GR activation prevents programmed cell death (apoptosis). Furthermore, in a mouse model of xenografted human breast cancer, glucocorticoid treatment and nuclear translocation of tumor GR is associated with resistance to chemotherapy-induced apoptosis and ultimately larger tumor growth (11, 12). Inhibition of apoptosis may also be driven by GR translocation to the mitochondria (33), consistent with the observed tendency toward greater variance in the relative subcellular distribution of GR between the cytoplasm and the nucleus. Since the GR has different mechanisms of activating signaling pathways in the nucleus versus non-nuclear locations, there are several possible mechanisms that might alter tumor growth in the mammary glands of isolated animals exposed to increased glucocorticoid action. Furthermore, the adrenergic components of the stress response on angiogenesis could further increase tumor growth (34).

Recently, a study of C3 (1)/SV40 large T-antigen (Tag) transgenic mice predisposed to developing invasive mammary gland carcinomas (35), revealed that social isolation in this species is also associated with increased glucocorticoid reactivity to an acute stressor as well as an increased tumor growth rate. In the SV40 T-antigen model, chronic social isolation from weaning was associated with significantly increased expression of genes encoding key metabolic pathway enzymes in the mammary gland before invasive cancer formation. Specifically, prolonged social isolation was associated with up-regulation of genes encoding key lipid synthesis and glycolytic pathway enzymes in the mammary tissues of 15-week-old mice, well before microscopic evidence of differences in invasive carcinoma between the two housing groups. In addition, gene expression differences reflecting inflammatory pathways were uncovered, supporting the hypothesis that differences in stress-induced inflammatory dysregulation (18) may also contribute to the susceptibility of genetically predisposed individuals to malignancy under conditions of chronic social isolation.

The majority (60%) of naturally occurring mammary tumors examined here expressed estrogen receptors (ER) and progesterone receptors (PR); the role of estrogens and progestins in mammary carcinogenesis and growth is well established (25). In this rat model, however, social isolation was associated with senescent ovaries at the age of tumorigenesis and growth; few isolated rats had hormonally active follicles or corpora lutea, (23), confirmed here by the condition of their vaginal epithelia. Therefore, ovarian function at best plays a permissive role, and likely does not account for the larger and more malignant tumors in isolates.

The most frequent neoplasia in these naturally occurring rat mammary tumors was ductal epithelial: premalignant intraductal hyperplasia (IDH), ductal carcinoma in situ (DCIS), and invasive ductal carcinoma (IDC). The spectrum of these diagnoses is also common in human breast cancer, suggesting that the Sprague–Dawley rat is an excellent model for human mammary cancer development and progression. However, equally important is the wide variety of spontaneously arising non-neoplastic mammary tumors, ''benign'' disease in which stromal tissue proliferated until it occupied as much as 54% of the female rat's body weight (M = 6.7%) and became a significant metabolic and physical burden. This mammary gland disease diversity provides the opportunity to study the psychosocial mechanisms that might affect whether hyperplasias ultimately follow a malignant versus benign trajectory.

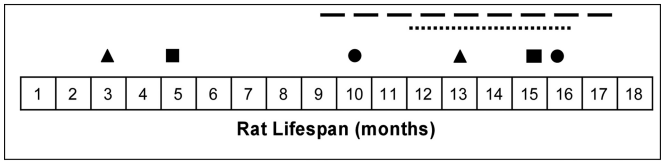

Fig. 5. Experimental protocol of biobehavioral assays: ▲ Corticosterone stress responses; ■. Exploration stress responses: ● vigilance testing, ····· ovarian cyclicity, and – – – — tumor palpation.

In summary, our model for investigating environmentally initiated psychosocial effects of mammary cancer initiation and growth provides a complementary approach to the current emphasis on inherited and spontaneous genetic alterations leading to changes in gene expression. First, it is a model of spontaneous tumors, which recapitulate most malignant and benign tumors in humans more accurately than the more expedient models using transgenes or carcinogens to stimulate rapid cancer growth or xenografts in which human cancer cells are injected into immunodeficient mice. Because this is a life span animal model of disease that examines dynamic interactions between the organism and its environment over a relatively long period, it illustrates not only the interaction between early stress responsiveness and later tumor pathology, but also tradeoffs between short-term benefits early in the life span, such as enhanced fertility of early ovarian maturity (23), yet increased disease burden at its end. Our data also point to the important and the somewhat unexpected role of the stress hormones in cancer biology. Specifically, in this animal model, adrenal dysregulation, not lengthening of estrogen or progesterone exposure, was associated with increased mammary tumor development and progression.

Interestingly, loneliness and stress vulnerability have been associated previously with gene expression changes in isolated ovarian cancer and immune cells in humans (36–38). This animal model is also another powerful tool for identifying the links in a network between specific aspects of social context, psychological states, neuroendocrine mechanisms, and tissue gene expression changes that culminate in benign hyperplasia or mammary cancer. Translating these findings into successful targets for intervention to reduce cancer risk will require transdisciplinary research that considers the diversity of human circumstance and the complexity of cancer biology (15, 39).

Materials and Methods

Female Sprague–Dawley rats (n = 40) were bred at Charles River Laboratories, Inc., weaned into same sex groups at 21 days of age, and shipped to our laboratory at 28 days of age (Fig. 5; pubertal data and methods are in ref. 23). In short, we randomly assigned 20 rats to socially isolated housing in a rack of single cages (26 × 23 × 22 cm) and 20 rats to four groups of five, each group in a large cage (46 × 61 × 36 cm). Cages had wire mesh floors over shared bedding pans and animals could smell and hear each other, but interact socially only in the group cages. All resided in the same colony room of an American Association for the Accreditation of Laboratory Animal Care accredited facility; the Institutional Animal Care and Use Committee of the University of Chicago approved all protocols. Detailed information for this and all methods are in *SI Text*.

Mammary Tumor Burden. In a series of bi-monthly health checks (Fig. 5), trained technicians palpated all mammary glands of middle-aged animals and noted the location and size of all nodules, using standard technique (23, 40). Estimates of tumor weight by palpation predicted the dissected tumor weight. Total tumor burden, the sum of all tumors within an animal, was determined at 15.0 ± 0.1 months of age, chosen for cross-sectional measurements to optimize the tradeoff between a longer opportunity to express the tumor phenotype and increasing mortality. At 15 months, 95% were still alive, 100% of animals that eventually developed a mammary

tumor had already done so, and 97.8% of those who were tumor-free remained so.

Tumor Diagnosis. Necropsy occurred later (18.8 ± 0.5 months of age; Fig. 5). Surgical pathologists specializing in breast cancer pathology diagnosed a randomly selected tumor subset from representative histological sections, classifying them (40) and categorizing them as malignant [2; including premalignant intraductal hyperplasia (41)], benign (1), or no tumor (0).

Hormone Receptor Status. Tissue microarrays (TMA) contained two different 1 mm cores per tumor. Primary antibodies were: anti-estrogen receptor α (C1355, 1:800, Millipore) anti-progesterone receptor (Ab 13, 1:200, Lab Vision Corporation) and anti-glucocorticoid receptor (3D5, 1:400, Abcam Inc.). After incubation with HRP-labeled polymer, reactions were completed with the Envision detection system using 3–3′ diaminobenzidine as the chromogen (DakoCytomation; staining intensity was excellent for GR, ER, and PR (all median and modal values = 3 on a 3-point scale).

In this study of GR in rat mammary tissue, we scored the cytoplasm and nucleus of both epithelial and the stromal cells [% cells with positively GR stain, coded as: (0) negative stain, (1) 5% (range 1 to 10% of cells), (2) 30% (range 11–50%), (3) 65% (range 51–80%), and (4) 90% (81%–100%)]. Following standard clinical method for assessing ER and PR status of mammary tumors and cancers, we recorded their presence or absence in the nuclei of epithelial and stromal cells.

Corticosterone Response to Stressors. At the beginning of behavioral night (lights-on), rats were stress-tested in an adjacent room. At 2 months of age, they spent 30 min in an unfamiliar cage scented with predator (fox) urine (Fig. 5) and at 13 months of age, 30 min in an unfamiliar restraint tube (Harvard Apparatus). Tail-blood samples were taken within 2 min to measure the adrenal stress hormone corticosterone at: prestress baseline, after 30 min of imposed stress and during recovery, 1 and 2 h after returning to their home cage. Serum concentrations of corticosterone were assayed by RIA (ICN Biomedicals), with slight modifications to increase sensitivity (intra-assay coefficient of variance = 9.4%; inter-assay variance = 8.1)

Ovarian Senescence. Vaginal cytology were analyzed daily (12–16 months of age; Fig. 5), quantifying estrogenization levels, cycle length, and reproductive state (22).

Behavioral Response to Stressors. Exploration in an unfamiliar environment is a classic stressor for rodents (42). We modified the technique to avoid arousal confounds by gently placing each animal in the home corner, important protection for this thigmotactic species, seated in a heavy ceramic bowl serving as a home base from which to remain vigilant or explore. A detailed ethological analysis was used to measure: exploration stress, anxiety, and boldness. Similar exploration responses to opening the home cage were quantified by the latency to emerge and touch the cage rim.

Statistical Analysis. Statistical analyses were conducted with Statview (SAS Institute; NS = not significant ($P > 0.05$, two-tailed tests), all means ± SEM.). Log- transformed tumor weights, arcsine transformed arcsine [sqrt (p)] percentages and z-scores met parametric distribution requirements.

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